Vo et al., Afr J Tradit Complement Altern Med. (2015) 12(4):-79-83
http://dx.doi.org/10.4314/ajtcam.v12i4.12

QUANTITATIVE ESTIMATION OF GINSENOSIDES IN DIFFERENT AGES OF *PANAX VIETNAMENSIS* AND THEIR ANTI-PROLIFERATION EFFECTS IN HELA CELLS

Hoang Tung Vo[1], Amal Kumar Ghimeray[2], Ngoc Thang Vu[3], Yeon-Ho Jeong[1*]

[1]Department of Medical Biotechnology, College of Biomedical Sciences, Kangwon National University, Chuncheon, South Korea, [2]Department of Biohealth Technology, College of Biomedical Sciences, Kangwon National University, Chuncheon, South Korea, [3]Faculty of Agronomy, Vietnam National University of Agriculture, Hanoi, Vietnam
Corresponding author: [1]* **Yeon-Ho Jeong E-mail:** jeongyhb@kangwon.ac.kr

Abstract

Background: The objective of study was to investigate the major compounds of ginsenosides in *Panax vietnamensis* in its growing stages and their anti-proliferative activity in HeLa cells.
Materials and Methods: The roots with different ages were analyzed by HPLC/MS. Anti-proliferation activity of *P. Vietnamensis* ginseng in HeLa cells was investigated using MTT assay.
Results: Three main compounds of ginsenoside, Rb1, Rg1 and majonoside R2 were increased with the increasing of age. However, ginsenoside Rc, Re, Rd and Rg3 did not show any alteration with growing age. The major compound majonoside R2 was accumulated maximum in 5 years old ginseng. According to the data, the anti-proliferation activity was directly proportional to the growing ages of ginseng. The eleven years old ginseng showed maximum effect than the younger roots. This effect may be related to the concentration of ginsenosides or majonoside R2 content in *P. Vietnamensis*.
Conclusion: The concentration of ginsenoside content in the roots of *P. Vietnamensis* is directly related to the growing ages. Overall, due to the higher concentration of ginsenoside in mature ginseng root, *Panax vietnamensis* ginseng can be used as an effective anti-proliferative drug.

Key words: *Panax vietnamensis*, anti-proliferation activity, HeLa cells, ginsenosides, ages

Introduction

Ginseng has been found and known as a traditional herbal medicine a long time ago. There are several kinds of ginseng such as *Panax ginseng* C.A Meyer (Korean ginseng), *Panax quinquefollius* L. (American ginseng), *Panax notoginseng* and *Panax vietnamensis* (Vietnamese ginseng). All kinds of ginseng are healthy foods and have many effects to medical therapeutic treatment such as antioxidant (Kim et al., 2011; Ramesh et al., 2012), anti-inflammatory (Hong et al., 2011), anti-tumor and anti-cancer (Sun et al., 2010; Lin et al., 2015; Lee et al., 2010; Lee et al., 2014; King et al., 2010; Yoon et al., 2010; Ji et al., 2012; Wang et al., 2008), etc.

Panax vietnamensis (P. Vietnamensis), commonly name as Vietnamese ginseng (VG), was found at Ngoc Linh Mountain in Vietnam in 1973. It is a special Panax species since it was discovered in the South while most of them are commonly found in the North (Le et al., 2014). *P. Vietnamensis* is proven to produce not only positive medical impacts but also beneficial psychological effects such as anti-stress, anti-depression as well as *in vitro* and *in vivo* anti-oxidation etc. (Duong et al., 2012).

Ginsenosides are the main bioactive compounds in ginseng. They are categorized into two groups: protopanaxdiol (PPD) and protopanaxtriol (PPT). Besides ginsenosides, VG contains ocotillol-type saponins, such as majonosides R1, R2 and vina-ginsenosides R1 and R2 (Duong et al., 2012; Nguyen et al., 1993). Recently, some studies have shown the significant effects of majonoside R2 on the central nervous system such as anti-stress, anti-depressive, and anxiolytic activities (Huong et al., 1995,1996,1997a,b,1998,2005). It was also demonstrated as an effective anti-tumor and anti-cancer agent. Konoshima et al., 1999 found that majonoside R2 exhibited potent anti-tumor-promoting activity on two-stage carcinogenesis test of mouse hepatic tumor using *N*-nitrosodiethylamine (DEN) as an initiator and phenobarbital (PB) as a promoter.
In ginseng, the contents of saponins or ginsenoside compounds varied with plant parts, growing environment and harvesting time or maturity. In this research, we tried to investigate and quantify the ginsenosides and majonoside content in different growing stages (ages) in VG. Furthermore, we also studied the anti-proliferative effect of VG in human cervical cancer cells (HeLa cells).

Materials and Methods

Sample collection and Materials

The roots (1 to 6 and 11 years old) of Vietnamese ginseng (VG) were collected from Ngoc Linh Mountain in Kontum Province, Vietnam in August, 2014. Ginsenoside Rb1, Rc, Rd, Re, Rg1, Rg3 standards were purchased from HWI ANALYTIK GmbH, Germany. Majonoside R2 was gifted from Institute of Drug Quality Control, Ho Chi Minh City, Vietnam. All other reagents were of analytical grade chemicals used in the experiment. Human embryonic kidney (HEK293) cells and HeLa cells were gifted from Plant Molecular Lab, Department of Medical Biotechnology, Kangwon National University, South Korea.

Sample preparation

The roots of VG were washed and cleaned, cut as slice and dried in dry oven at 50°C. The dried VG were crushed and passed through a 40 mesh sieve to collect the VG powder.200mg of each different age of VG was dissolved in 2ml of 80% Methanol and sonicated for 5 hrs at room temperature. The samples were filtered (0.45µm) and condensed in a rotary evaporator to get powder extract.

HPLC quantification of ginsenoide

To quantify the ginsenoide content in the different age Vietnamese ginseng, high-performance liquid chromatography (HPLC) system (CBM-20A; Shimadzu Co, Ltd., Kyoto, Japan) with 2 gradient pump systems (LC-20AT; Shimadzu, Japan), an auto sample injector (SIL-20A; Shimadzu), a UV-detector (SPD-10A; Shimadzu) and a column oven (CTO-20A; Shimadzu) were used for analysis. The separation was performed on a C18 column (Synergi 4µ MAX-RY, 150 × 4.6 mm, 4 micron Phenomenex. Inc., Torrance, CA, USA). The binary mobile phase consisted of 0.1% formic acid in acetonitrile (B) and distilled water (A) was used for separation. The system was run with a gradient program: 0-2min: 0-5%, 2-7min: 5-50%, 7-15min: 50-80%, 15-17min: 80-5%, and iso-cratic at 5% (17-20min). The experiment was conducted at 35°C with the flow rate of 0.3 ml per minute. The injection volume of standards and samples was 2 µl.

Mass spectrometry

Mass spectrometry was performed on a Triple Quadrupole Mass (ThermoFisher, USA) equipped with electrospray ionization (ESI) probe. The TQM mass spectrometer was operated under the following parameters: negative ion mode, the spray voltage 3.5 kV, capillary voltage 10 V, capillary temperature 350°C. High-purity nitrogen (N_2) was used as sheath gas and the sheath pressure was 35 units. The auxiliary gas was N_2 and the pressure was 10 units. Argon was employed as collision gas at a pressure of 1.5 mTorr. Full scan of m/z ranging was carried out from m/z 50 to 2000.

Cell culture and cytotoxic test

HEK293 and HeLa cells were cultured in DMEM supplemented with 5% (by volume) heat-inactivated FBS and 1% antibiotic/antimycotic at 37°C in a humidified 5% CO_2 atmosphere. Approximately 3 x 10^5 cells were seeded on 100 mm diameter tissue culture plates (SPL Life Sciences, Korea) and the media was changed every 2 days. Cytotoxic effects were determined by the MTT assay. A total of 10^4 cells were plated per well in 96-well plates with 100 µl culture medium for 24 hrs and then exposed to 0.25, 0.5 and 1 mg/ml of sample for 48 hrs. After removing supernatant of each well, a total of 5 µl of MTT solution [10 mg/ml in phosphate-buffered saline (PBS)] was added to each well at the time of incubation. After 3 hrs of incubation, the supernatant was discarded and 200 µl of DMSO were added to each well to terminate the reaction. The absorbance was measured at 550 nm using an ELISA plate reader (Bio-Tek, Winooski, VT, USA).

Statistical analysis

All the data were expressed as the mean value ± standard deviation (SD) of each experimental group (n=3). The results were processed using Excel 2007 (Microsoft, Redmond, WA, USA).

Results and Discussion

Investigation of ginsenosides in different ages of *Panax Vietnamensis* ginseng

Table 1 shows the content of ginsenosides and majonoside in *P. Vietnamensis* roots harvested in different years. Six ginsenosides, Re, Rg1, Rb1, Rc, Rd and Rg3 (20(S)-Rg3, 20(R)-Rg3) and majonoside R2 were investigated by HPLC/MS.

According to data, Ginsenoside Rb1, Rg1, Re and majonoside R2 increased with the increasing of ages. Ginsenoside Rg1 increased continuously with age from 1 to 11 years. In 11 years ginseng, the Rg1 content was 2.40 times higher than that of 1 year old ginseng root. Likewise, majonoside R2 which is the main compound in VG was found to be highest in concentration. It increased slowly from 1 to 4 years old *P. Vietnamensis* (from 2.48 mg/ml to 3.00 mg/ml), but rapid increase was observed in 5 years old VG (8.08 mg/ml), which was 2.7 folds higher than 4 years old VG. After 5 years, majonoside R2 was increased slowly compare to its previous growing years. In 11 years old VG, ginsenoside contained was 10.49 mg/ml (30% increase than 5 years old VG). This data may suggest that the time to harvest VG can be 5 years to get good amount of MR2. Ginsenoside Rg1 and majonoside R2 increased continuously with ages. However, in case of ginsenoside Rb1, increment was observed up to 6 years old root (from 2.66 mg/ml to 4.33 mg/ml) but it was slightly decreased (4.10mg/ml) in 11 years old VG.

Ginenoside Re also increased with increase in years. The concentration of Re was found to be 0.160 mg/ml in the 1 year old root, whereas, its concentration was increased to 0.646 mg/ml in 11 years old VG. This increment was nearly 4.03 times higher than that of one year old VG. In case of other ginsenosides such as Rc, Rd and Rg3, no significant difference were observed in their contents according to their age. Their concentration was stable with age.

The total ginsenosides was increased gradually from one (7.73 mg/ml) to four years old (9.82 mg/ml) VG, but rapid increase was observed after 5 years (14.11mg/ml). The 11 years old VG showed 18.52 mg/ml total ginsenoside content which was nearly 2.39 times higher than one year old VG.

The change of ginsenosides with age of *P. Vietnamensis* ginseng is slightly different comparing to those of *Panax ginseng* (Shi et al., 2007) and *Panax quinquefolius* (Qu et al., 2009). For example, according to Shi et al., 2007, ginsenoside Rb1, Rg1 and Rd, the main compounds of *Panax ginseng* were increased with age but ginsenoside Re was decreased. However, ginsenoside Re and Rb1 were increased with age of *Panax quinquefolius* while ginsenoside Rc, Rd and Rg1 were not changed much (Qu et al., 2009). This variation may be due to the difference in *Panax* species and growing environment which causes alteration in phytochemical content in the plant.

Table 1: Ginsenoside contents in different ages of *Panax vietnamensis* (all ginsenoside concentration are mg/ml).

Ages	Re	Rg1	MR2	Rb1	Rc	Rd	20(S)-Rg3	20(R)-Rg3	Total
1	0.160±0.001	0.513±0.004	2.487±0.021	2.660±0.023	0.014±0.001	1.844±0.011	0.052±0.001	0.000±0.001	7.730±0.063
2	0.157±0.001	0.627±0.005	2.640±0.022	2.792±0.022	0.006±0.001	1.878±0.013	0.053±0.001	0.007±0.001	8.160±0.065
3	0.366±0.003	0.703±0.005	2.393±0.02	3.999±0.03	0.045±0.001	1.749±0.01	0.080±0.001	0.015±0.001	9.350±0.071
4	0.176±0.001	0.724±0.005	3.006±0.025	4.180±0.035	0.025±0.001	1.631±0.01	0.054±0.001	0.025±0.001	9.821±0.079
5	0.003±0.001	0.878±0.006	8.088±0.05	3.316±0.031	0.010±0.001	1.797±0.01	0.021±0.001	0.005±0.001	14.117±0.101
6	0.683±0.005	0.927±0.007	8.127±0.05	3.439±0.033	0.007±0.001	1.486±0.01	0.016±0.001	0.008±0.001	14.694±0.108
11	0.646±0.005	1.236±0.009	10.494±0.07	4.103±0.037	0.026±0.001	1.989±0.021	0.021±0.001	0.011±0.001	18.525±0.145

Anti-proliferation effect

Human embryonic kidney (HEK293) cells were used as normal cell as a positive control. The proliferation of HEK293cells was shown in Fig 1. The treatment of sample to HEK293 cell did not show any negative effect. The cell growth was increased with the increasing of age of VG. This could be due to high amount of ginsenosides present in the sample may help the growth of HEK293 cells. The result showed that the VG is not toxic for normal cells.

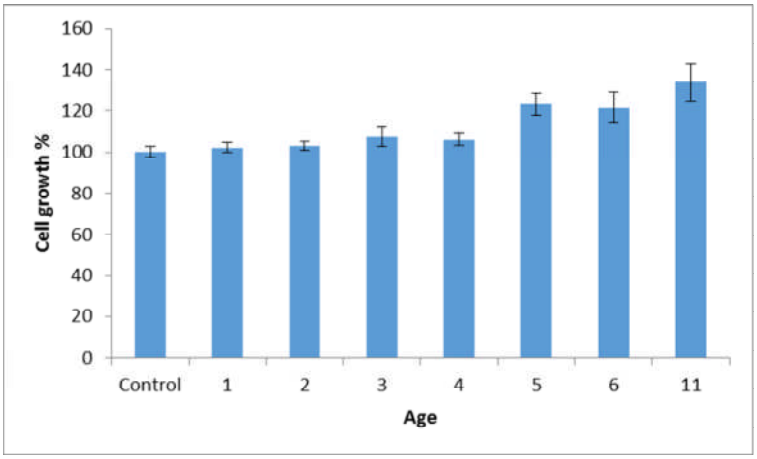

Figure 1: Effects of *Panax vietnamensis* with different ages in HEK293 cells

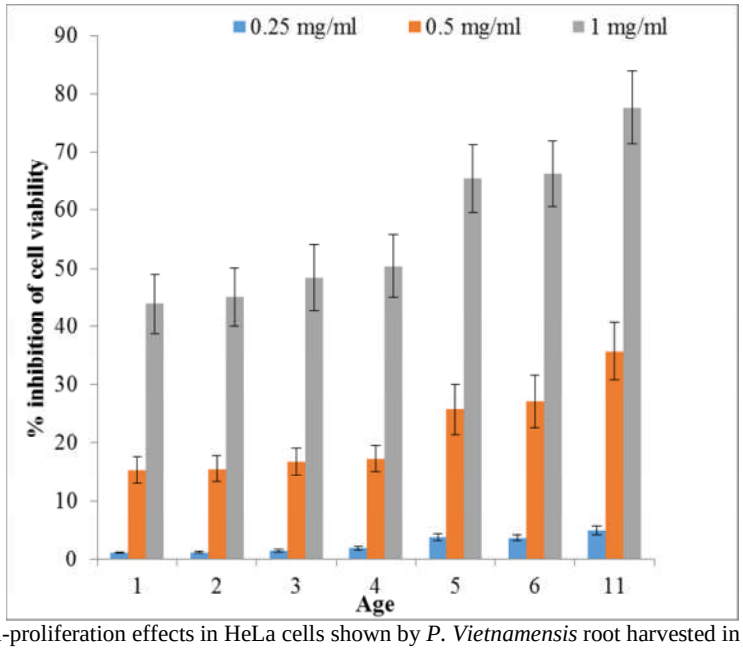

Figure 2:. Anti-proliferation effects in HeLa cells shown by *P. Vietnamensis* root harvested in different years

The proliferation of HeLa cells with and without the treatment VG were shown in Fig 2. The inhibition percentage of cell growth was rapidly increased upon the concentration of samples and the age of the sample used in the experiment. The highest effect was observed with 11 years old (77.7% inhibition of cell viability) VG. As previous studies, ginsenoside Rg3 might cause to the anti-proliferation activity in HeLa cells (Lee et al., 2010; Lin et al., 2015). However, the concentration of ginsenoside Rg3 in the raw *Panax vietnamensis* ginseng is too low and may not effect to the anti-proliferation of raw VG. We also did the MTT assays with 1 µg/ml of majonoside R2 standard and the result show 19% inhibition of cell viability. Therefore, this effect may be due to the content of MR2 in the sample or could be due to the synergistic effect of the compounds present in the sample.

Conclusion

The concentration of ginsenoside content in the roots of *P. Vietnamensis* is directly related to the growing ages. The major ginsenosides (Re, Rg1, Rb1), and majonoside R2 concentrated more in mature roots (after 5 years). In the anti-proliferation bioassay, the VG inhibited HeLa cells in dose dependent manner. Finally, due to the higher concentration of ginsenoside in mature ginseng root, VG can be used as an effective anti-proliferative drug.

Acknowledgment

This work (Grants No. C0219332) was supported by Business for Cooperative R&D between Industry, Academy, and Research Institute funded by Korea Small and Medium Business Administration in 2014. This study was also supported by 2014 Research Grant from Kangwon National University (No. 120131276).

References

1. Duong, T.N., Nguyen, P.H., Hoang, X.C., Tran, C.L., Bui, T.V. and Lam, B.T. (2012). *In vitro* culture of petiole longitudinal thin cell layer explants of Vietnamese ginseng (*Panax vietnamensis* Ha et Grushv.) and preliminary analysis of saponin content. International Journal of Applied Biology and Pharmaceutical Technology. 3(3): 178-190.
2. Hong, C.E. and Lyu, S.Y. (2011). Anti-inflammatory and Anti-oxidative Effects of Korean Red Ginseng Extract in Human Keratinocytes. Immune Netw. 11: 42–49.
3. Huong, N.T., Matsumoto, K. and Watanabe, H. (1998). The anti stress effect of majonoside-R2, a major saponin component of Vietnamese ginseng: neuronal mechanisms of action. Methods Find Exp Clin Pharmacol. 20:65–76.
4. Huong, N.T., Matsumoto, K., Kasai, R., Yamasaki, K., Duc, N.M., Nguyen, T.N. and Watanabe, H. (1995). Crude saponin extracted from Vietnamese ginseng and its major constituent majonoside-R2 attenuate the psychological stress- and foot-shock stress-induced antinociception in mice. Pharmacol Biochem Behav. 52: 427-432.
5. Huong, N.T., Matsumoto, K., Yamasaki, K. and Watanabe, H. (1997). Majonoside-R2 reverses social isolation stress-induced decrease in pentobarbital sleep in mice: possible involvement of neuro active steroids. Life Sciences. 61: 395-402.

6. Huong, N.T., Matsumoto, K., Yamasaki, K., Duc, N.M. and Watanabe, H. (1997). Majonoside-R2, a major constituent of Vietnamese ginseng, attenuates opioid-induced Antinociception. Pharmacol Biochem Behav. 57: 285-291.
7. Huong, N.T., Matsumoto, K., Yamasaki, K., Duc, N.M., Nham, N.T. and Watanabe, H. (1996). Effects of majonoside-R2 on pentobarbital sleep and gastric lesion in psychologically stressed mice. Pharmacol Biochem Behav. 53: 957-963.
8. Huong, N.T., Murakami, Y., Tohda, M., Watanabe, H. and Matsumoto, K. (2005). Social isolation stress-induced oxidative damage in mouse brain and its modulation by majonoside-R2, a Vietnamese ginseng saponin. Biol Pharm Bull. 28:1389–1393.
9. Ji, Y., Rao, J., Cui, J., Bao, H., Chen, C., Shu, C. and Gong, J.R. (2012). Ginsenosides extracted from nanoscale Chinese white ginseng enhances anticancer effect. J Nanoscience and Nanotechnology. 12:1-5.
10. Kim, H.G., Yoo, S.R., Park, H.J., Lee, N.H., Shin, J.W., Sathyanath, R., Cho, J.H. and Son, C.G. (2011). Antioxidant effects of *Panax ginseng* C.A. Meyer in healthy subjects: a randomized, placebo-controlled clinical trial. Food Chem Toxicol. 49: 2229-35.
11. King, ML and Murphy, L.L. (2010). Role of cyclin inhibitor protein p21 in the inhibition of HCT116 human colon cancer cell proliferation by American ginseng (*Panax quinquefolius*) and its constituents. Phytomedicine. 17:261-268.
12. Konoshima, T., Takasaki, M., Ichiishi, E., Murakami, T., Tokuda, H., Nishino, H., Duc, N.M., Kasai, R. and Yamasaki, K. (1999). Cancer chemopreventive activity of majonoside-R2 from Vietnamese ginseng, *Panax vietnamensis*. Cancer Lett. 147: 11-6.
13. Le, T.H.V., Lee, S.Y., Kim, T.Y., Kim, J.Y., Kwon, S.W., Nguyen, N.K., Park, J.H. and Nguyen, M.D. (2014). Processed Vietnamese ginseng: Preliminary results in chemistry and biological activity. J Ginseng Res. 38: 154-159.
14. Lee, K.A., Kim, K.T., Chang, P.S. and Paik, H.D. (2014). *In vitro* cytotoxic activity of ginseng leaf/stem extracts obtained by subcritical water extraction. J Ginseng Res. 38: 289-292.
15. Lee, W.H., Choi, J.S., Kim, H.Y., Park, J.H., Park, B.D., Cho, S.J., Lee, S.K., Surh, Y.J. (2010). Potentiation of etoposide-induced apoptosis in HeLa cells by co-treatment with KG-135, a quality-controlled standardized ginsenoside formulation. Cancer Lett. 294:74-81.
16. Lin, Y., Jiang, D., Li, Y., Han, X., Yu, D., Park, J.H. and Jin, Y.H. (2015). Effect of sun ginseng potentiation on epirubicin and paclitaxel-induced apoptosis in human cervical cancer cells. J Ginseng Res. 39: 22-28.
17. Nguyen, M.D., Nguyen, T.N., Kasai, R., Ito, A., Yamasaki, K. and Tanaka, O. (1993). Saponins from Vietnamese ginseng, Panaxvietnamensis Ha etGrushv. Collected in central Vietnam. I. Chem Pharm Bull. 41: 2010-4.
18. Qu, C., Bai, Y., Jin, X., Wang, Y., Zhang, K., You, J. and Zhang, H. (2009). Study on ginsenosides in different parts and ages of *Panax quinquefolius L*. Food Chem. 115: 340-346.
19. Ramesh, T., Kim, S.W., Hwang, S.Y., Sohn, S.H., Yoo, S.K. and Kim, S.K. (2012). Panax ginseng reduces oxidative stress and restores antioxidant capacity in aged rats. Nutr Res. 32: 718-726.
20. Shi, W., Wang, Y., Li, J., Zhang, H. and Ding, L. (2007). Investigation of ginsenosides in different parts and ages of *Panax ginseng*. Food Chem. 102: 664-668.
21. Sun, S., Wang, C.Z., Tong, R., Li, X.L., Fishbein, A., Wang, Q., He, T.C., Du, W. and Yuan, C.S. (2010). Effects of steaming the root of *Panax notoginseng* on chemical composition and anticancer activities. Food Chem. 118: 307-314.
22. Wang, L.B., Wu, Z.H., Gao, H.Y., Huang, J., Sun, B.H. and Hu, L.J. (2008). A new compound with cytotoxic activities from the leaves of *Panax ginseng* C.A. Meyer. Chinese Chem Lett. 19:837-840.
23. Yoon, S.R., Lee, G.D., Park, J.H., Lee, I.S. and Kwon, J.H. (2010). Ginsenoside composition and antiproliferative activities of explosively puffed ginseng (*Panax ginseng* C.A. Meyer). J Food Sci. 75: 378-82.